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13. ABSTRACT (Maximum 200 words) The objective of the project is to establish and use an *in vitro* liver model from rodents and humans to develop quantitative data on the metabolism of toxic chemicals. In the third year of the project, liver slices from rat and human liver were compared to evaluate their capacity for chloroform metabolism. It was observed that the weight (an indication of the slice thickness) of the liver slices was inversely related to the rate of chloroform metabolism, suggesting that metabolism was limited by diffusion into the tissue in the thicker slices. Using the thinnest slices possible, the kinetic constants for chloroform metabolism by rat and human liver slices were determined. The V_{max} values for chloroform metabolism were 2.82 ± 0.79 nmol/min/g tissue and 2.91 ± 0.99 nmol/min/g tissue, with rat and human liver, respectively and the K_m values were 25.5 ± 18.4 nmol/flask and 8.33 ± 1.9 nmol/flask, rat and human liver, respectively. Rat hepatocytes incubated under similar conditions metabolized chloroform with a V_{max} of 10 nmol/min/g and a K_m of 93 nmol/flask. In summary, the liver slice system was readily adaptable to investigation of the metabolism of volatile chemicals.

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IN VITRO SYSTEM FOR STUDYING METABOLISM OF ENVIRONMENTAL CHEMICALS IN HUMAN CELLS

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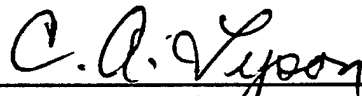_Carol E. Green_

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LIST OF PERSONNEL

Carol E. Green   Principal investigator.

Jack E. Dabbs   Project leader: hepatocyte isolations, liver slice
preparation, incubations, gas chromatography analyses.

OBJECTIVES

The objective of the project is to establish and use an *in vitro* system of intact isolated cells from rodent and human tissues to develop quantitative data on the metabolism of toxic chemicals that can be used for risk assessments. The following halogenated aliphatic solvents are being studied: chloroform, 1,1,1-trichloroethane, trichloroethylene, dichloromethane, bromochloromethane, and carbon tetrachloride. Chloroform is being used to establish the conditions with rat liver preparations for the generation of kinetic constants for metabolism, which are determined as disappearance of the parent compound. Isolated hepatocytes and precision-cut liver slices are being compared to optimize the correspondence between the *in vitro* results obtained and the published *in vivo* data. The system developed with rat liver is now being applied to human liver incubations. The same set of halogenated solvents is being studied, and the resulting data should allow quantitative comparison of the metabolism and cytotoxicity in these species. These data will be analyzed to characterize interspecies differences in the kinetics of metabolism.

MATERIALS AND METHODS

HEPATOCYTE ISOLATION

Hepatocytes were isolated from male F344 rats (300-450 g) by the whole-liver perfusion method (Green et al., 1983). The cell yield and viability were determined by counting the cell suspension using a hemacytometer and calculating the percentage of cells that excluded trypan blue. Hepatocytes were also isolated from human liver specimens, using the biopsy perfusion technique (Allen and Green, 1993).

Isolated hepatocytes were suspended in a modified Waymouth's 752/1 culture medium (CMH) that contained 11.2 μg/ml alanine, 12.8 μg/ml serine, 24.0 μg/ml asparagine, 84.0 μg/ml gentamicin sulfate, 0.168 μg/ml aminolevulinic acid, 5.0 μg/ml oleic acid, 5.0 μg/ml linoleic acid, 1.0 μg/ml D,L-tocopherol, 288 ng/ml testosterone, 272 ng/ml estradiol, 393 ng/ml dexamethasone, 7.9 μg/ml thyroxin, 30 ng/ml glucagon, 0.02 U/ml insulin, and 0.2% BSA.

PREPARATION OF PRECISION-CUT LIVER SLICES

Liver cores were cut [prepared] from either rat liver or a dissected lobe of the human liver by using a sharpened cylindrical tube attached to a drill press. A core (about 2 cm in length) was then placed in the tissue holder of the Krumdieck slicer. Slices were prepared with a diameter of about 1 cm and a thickness of approximately 250-300 μm. Two or four slices were loaded onto each mesh insert and placed in scintillation vials for a preincubation period at 37°C for 1 hr, using the same culture medium as the hepatocytes. The medium was then aspirated and replaced with fresh medium (2.0 ml/vial).

INCUBATION OF HEPATOCYTES AND LIVER SLICES WITH HALOGENATED SOLVENT

The hepatocytes were prepared at a density of 4×10^6/ml, and 4 ml of cell suspension was added to each side-arm incubation flask. The flasks were matched for total volume (about 25 ml) and outfitted with gas-tight valves to allow repeat sampling of either the head space or the medium (Mininert valves, Pierce, Rockford, IL) to fit both the top and side-arm opening of the flask. After addition of the cells, the flasks were gassed

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vigorously for about 30 s with 95% air:5% CO_2 and immediately stoppered. The solvent (chloroform in the studies described in this report) was added with a Hamilton syringe through the Mininert valve directly into the medium. The flasks were placed in an oscillating water bath and maintained at 37°C at 60-70 osc/min.

The liver slices (4 slices per vial or between 130-200 mg tissue) were placed on screen mesh support and inserted into a scintillation vial. The vials contained 2 ml of the same medium used with isolated hepatocytes and were gassed with 95% air:5% CO_2. They were placed in a rotating rack at 37°C; the rack allowed the slices on the support mesh to roll freely through the medium. After a preincubation period of 1 hr, the medium was changed, the flasks gassed again, and the test chemical (chloroform) was added through the gas-tight valve.

The stock solutions of chloroform were prepared by dissolving the halogenated hydrocarbon in DMSO and then diluting it into CMH in a sealed vial. Stock solutions were prepared to give the following concentrations of chloroform. The actual concentrations of these stock solutions were determined so that the dose of chloroform to the entire flask could be estimated. Aliquots of these solutions were added directly to the media of the incubation vessels (0.1 ml to hepatocytes and 0.05 ml to liver slices).

GAS CHROMATOGRAPHIC ANALYSIS OF CHLOROFORM   ---

Chloroform was analyzed using gas chromatography with a 2 mm × 6 ft glass column packed with 0.1% SP1000 on 80/100 Carbopack C using a Varian Model 3700 gas chromatograph equipped with an electron capture detector and a Hewlett-Packard model 3390A integrator. The following parameters were used: column temperature, 125°C; detector temperature, 150°C; injector, 250°C; N_2 flow rate, 30 ml/min.

A standard curve for chloroform was prepared by adding 10 μl of chloroform to 4.9 ml of DMSO in a 1-dram vial with a Teflon septum. An aliquot (10 μl) was further diluted with 1.99 ml of methanol. Standard solutions for injection were prepared by diluting 20, 60, 125, and 200 μl to a volume of 2 ml with methanol in septum-sealed 0.5-dram vials to yield 0.15 to 1.5 ng of chloroform per microliter. These solutions were injected directly into the gas chromatograph.

STATUS OF RESEARCH EFFORT

BACKGROUND AND RATIONALE

The purpose of the current experiments is to extend previous work conducted at SRI on the cytotoxicity and metabolism of chlorinated solvents (Tyson et al., 1983; Knadle et al., 1990). In those studies, the dose-response relationship for cytotoxicity of several chlorinated solvents to rat hepatocytes was established (Tyson et al., 1983), using relatively high concentrations and short exposure periods. For example, the EC50 (concentration at which 50% of the cellular LDH was released to the medium in 2 hr) for chloroform was calculated to be 7.1 ± 0.49 mM. At these concentrations, a decrease in chlorinated hydrocarbon level in the medium was not detected.

The metabolite profile of another chlorinated hydrocarbon, trichloroethylene, was well-characterized by our laboratory using rat and human hepatocytes (Knadle et al., 1990) to compose the disposition of this solvent and to use the formation of particular metabolites as a means for predicting hepatocarcinogenic risk to humans. In that study, hepatocytes from both species metabolized trichloroethylene to trichloroethanol and its glucuronide, chloral hydrate, and trichloroacetic acid.

The rate and extent of metabolism was greater in rat than in human hepatocytes. The pattern of metabolites also varied with species in our study. Rat hepatocytes formed proportionally more trichloroacetic acid, the metabolite believed to be responsible for the hepatocarcinogenicity of trichloroethylene (Elcombe, 1985). Although kinetic constants were not calculated from the experiments with trichloroethylene, it was apparent that metabolism by rat hepatocytes became saturated at a higher concentration than by human hepatocytes.

Our goal in the current research project is to establish an *in vitro* technique for readily determining kinetic constants for metabolism of chlorinated solvents, using tissues from both laboratory species and humans. Although we initially planned to use isolated hepatocytes in suspension for these studies, new data led us to consider whether the basic *in vitro* model for these studies should be modified. We have observed in other experiments that the viability of human hepatocytes in suspension culture decreases much faster than the viability of rat liver cells. On the average, rat hepatocytes leak about 5%-10% of the total LDH into the extracellular medium during a 4-hr incubation period. Human hepatocytes lose at least 20% of the total LDH to the medium and some preparations release as much as 60%-80% in 4-hr.

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This observation led us to conduct a study that compared the metabolism of a test compound, mofezolac, by suspension and monolayer cultures of human hepatocytes (Green and LeValley, 1993). In that study, human hepatocyte monolayer cultures metabolized mofezolac with a higher Vmax (similar to that obtained with human liver microsomes) and formed a more complete metabolite profile than human hepatocyte suspension cultures. In contrast, no difference was found in the rate of benzo(a)pyrene metabolism by rat hepatocytes in suspension and monolayer cultures (Knadle et al., 1992).

Unfortunately, monolayer cultures are probably not a practical alternative system for the present studies. Volatile compounds are very difficult to handle in monolayer cultures because the halogenated solvents dissolve into the plastic of the culture dishes and hepatocytes do not attach to glass culture vessels. Therefore, we decided to investigate precision-cut liver slices, an alternative *in vitro* system that has been developed as a model for metabolism studies and reported to be useful with human and rat liver specimens.

Precision-cut liver slices have several advantages over hepatocytes, including intact cell connections between both parenchymal and nonparenchymal cells, stability of function and viability in cold storage for at least 24 hr, and a relatively simple preparation method that can be used with pieces of tissue that cannot be perfused to isolate cells. Unfortunately, we observed a disadvantage of liver slices when we compared the rate of metabolism of a model cytochrome P450 substrate, 7-ethoxycoumarin (Green et al., 1994). Ethoxycoumarin O-deethylase activity was significantly higher in both rat and human hepatocyte cultures than in liver slices. Nevertheless, the scientific and practical advantages of the tissue slice system encouraged us to continue investigating this model and attempt to optimize it for studies of chlorinated solvents.

LIVER SLICES

The optimal thickness recommended for precision-cut liver slices is 200 to 250 μm thick, because tissue viability is well maintained and the ratio of damaged cells on the cut surfaces to intact interior cells is minimized under these conditions (Brendel et al., 1993). However, a recent report suggested that liver slices for metabolism studies should be as thin as possible. Dogterom (1993) found that the drug metabolism was essentially the same in incubations containing slices varying in thickness from approximately 125 to 750 μm, suggesting that only the cells on the outer surface of the slice were active in metabolizing the substrates. Since our preliminary experiments suggested that the

metabolism of chloroform by rat liver slices was slower than metabolism by rat hepatocytes, we decided to determine the influence of slice thickness on the loss of chloroform from the incubations.

As a measure of slice thickness, we determined the wet weight of the slices. The thinnest rat liver slices that could be made with our Krumdieck slicer averaged 25 mg wet weight. Figure 1 shows the results of an experiment relating the rate of chloroform loss to the wet weight of slices between 25 and 150 mg each. In agreement with Dogterom (1993), we found that the rate of metabolism decreased significantly as the weight of the slice increased. In fact, we observed that the total loss of chloroform was essentially the same in all the incubations regardless of the slice weight. As a result, in all future experiments with liver slices, we are preparing the thinnest slices possible.

The Lineweaver-Burk plots of data from three rat liver slice experiments and three human liver slice experiments are presented in Figures 2 and 3, respectively. Table 1 lists the K_m and V_{max} values that were calculated from these experiments. There were no significant differences in the kinetic constants calculated for the two species. However, the rat liver slice experiments were performed before the experiment to determine the effect of slice weight on the rate of chloroform metabolism and so thicker slices were used with rat liver than with human liver. As a result, additional rat liver slice experiments are in progress to verify the results. An abstract summarizing this research on the metabolism of chloroform by rat and human liver slices has been submitted for presentation at the North American International Society for the Study of Xenobiotics to be held in October 1994 (see the Appendix).

Another experiment was performed with human liver slices to determine the effect of cold storage of the slices on the metabolism of chloroform. Fisher et al. (1994) reported that liver slices can be stored at wet ice temperatures in organ preservation solution for at least 48 hr without significant loss of viability or function. With one human liver specimen, H90, an experiment was performed on the day that the liver was received and repeated the next day after storing the slices in organ preservation solution (UW from Du Pont) at approximately 4°C for 24 hr. The results in Figure 4 demonstrate essentially no difference in the metabolism of chloroform in the two experiments. This cold storage of liver slices is a significant practical advantage making it possible to complete more experiments with a single human liver specimen.

Preliminary experiments were also conducted using carbon tetrachloride to establish the analytical techniques and the appropriate concentrations for detecting metabolism by liver preparations. In general the rate of metabolism of this compound seemed to be lower than the rate for chloroform. The Lineweaver-Burk plot of the results of this experiment, presented in Figure 5, show that the rate of carbon tetrachloride

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metabolism was much lower than that of chloroform The kinetic constants calculated from this first experiment with carbon tetrachloride were V_{max} = 0.014 nmol/min/g tissue and K_m = 0.15 nmol/flask. The results from *in vivo* experiments using gas uptake data to determine metabolic constants also indicate that carbon tetrachloride is metabolized at a lower rate than other chlorinated solvents (Gargas et al., 1986).

HEPATOCYTES

An experiment was performed using the same incubation conditions and experimental protocol worked out for liver slices. Figure 6 shows the Lineweaver-Burk plot of these data. The V_{max} calculated from the results is 10 nmol/min/g tissue and the K_m was 93.0 nmol/flask. We determined that the average wet weight of 10^6 rat hepatocytes is 13 mg. Using this figure, the V_{max} obtained with rat hepatocytes can be converted to allow comparison with the liver slice data, 18.5 nmol/min/g, suggesting that hepatocytes metabolize chloroform at a higher rate than liver slices. This result agrees with our observations on the metabolism of 7-ethoxycoumarin and 1,6-dinitropyrene (Green et al., 1994).
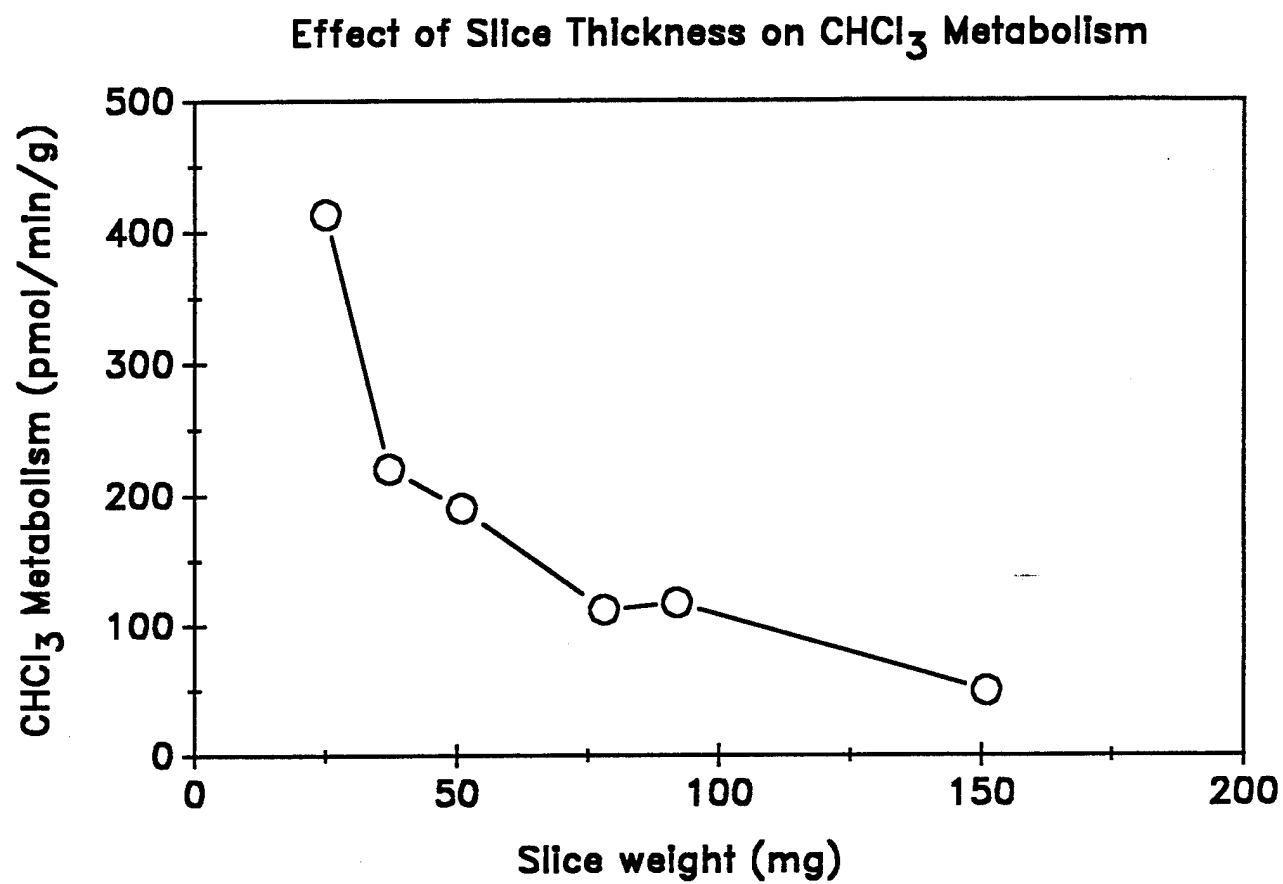
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Effect of Slice Thickness on CHCl$_3$ Metabolism

Figure 1.   The effect of rat liver slice weight on chloroform disappearance from the headspace.

CHCl₃ Metabolism by Rat Liver Slices (11/24/93)

$b = 5.27 \times 10^{-4}$
$m = 8.82 \times 10^{-3}$

CHCl₃ Metabolism in Rat Liver Slices (12/1/93)

$b = 3.06 \times 10^{-4}$
$m = 4.05 \times 10^{-3}$

CHCl₃ Metabolism in Rat Liver Slices (12/9/93)
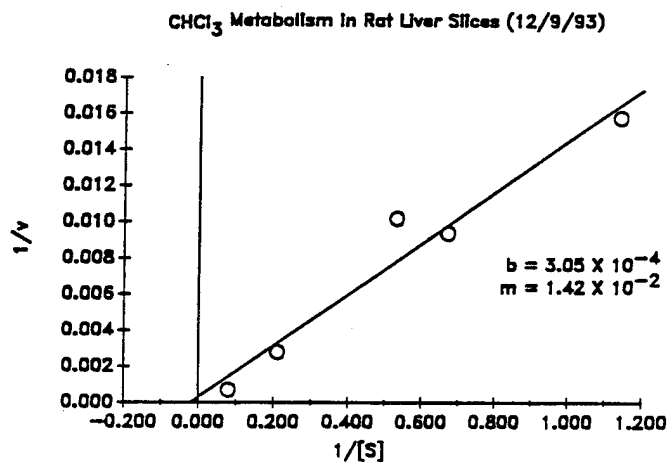
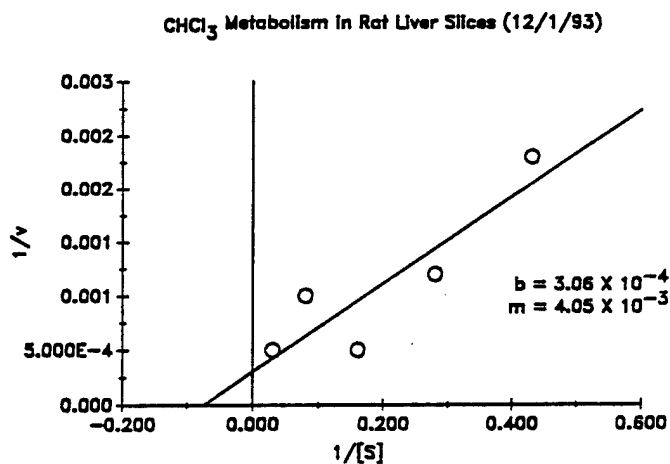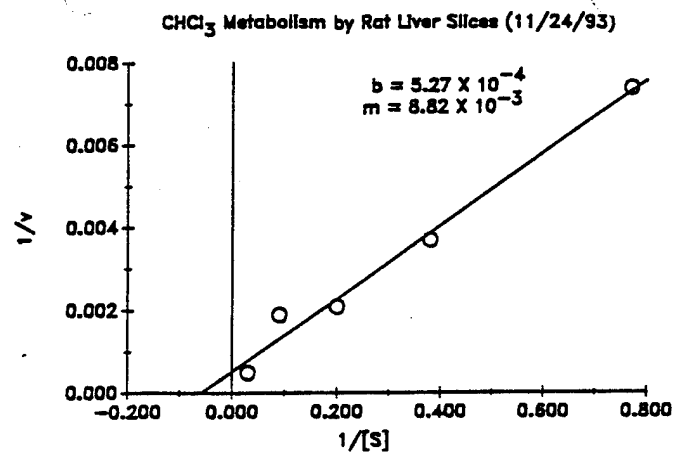$b = 3.05 \times 10^{-4}$
$m = 1.42 \times 10^{-2}$

Figure 2. Lineweaver-Burk plots of three replicate rat liver slice experiments in which the rate of chloroform disappearance from the flask headspace was determined.
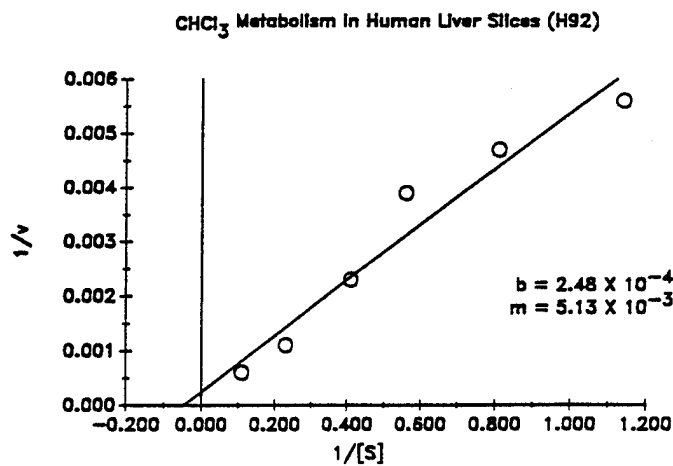
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CHCl$_3$ Metabolism in Human Liver Slices (H89)

b = 4.61 X 10^{-4}
m = 2.91 X 10^{-3}

CHCl$_3$ Metabolism in Human Liver Slices (H90)

b = 3.97 X 10^{-4}
m = 3.44 X 10^{-3}

CHCl$_3$ Metabolism in Human Liver Slices (H92)

b = 2.48 X 10^{-4}
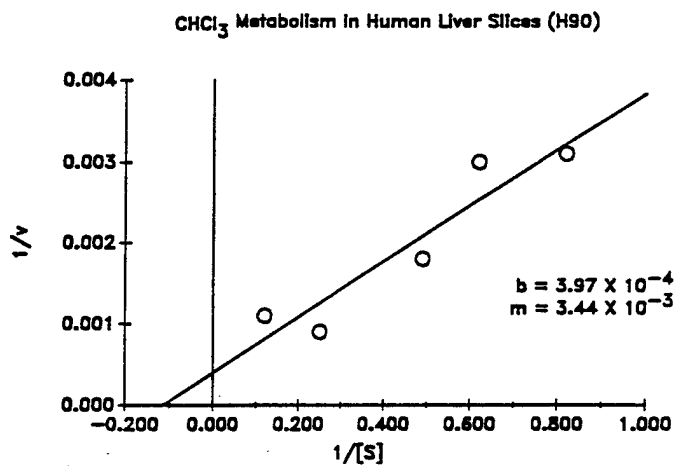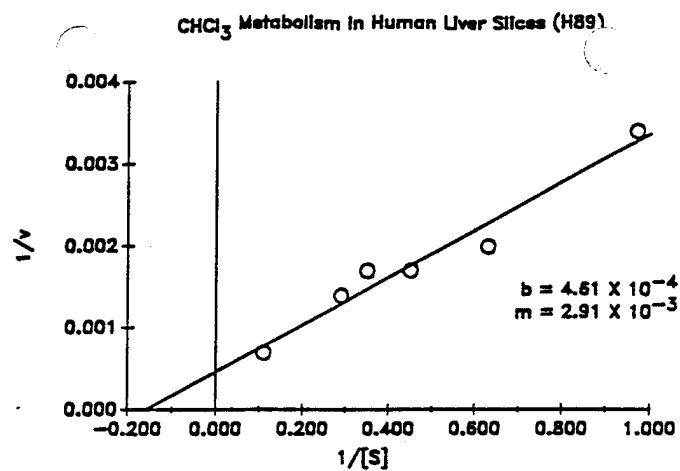m = 5.13 X 10^{-3}

Figure 3.   Lineweaver-Burk plots of three replicate human liver slice experiments in which the rate of chloroform disappearance from the flask headspace was determined.

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Table 1
KINETIC CONSTANTS OF CHLOROFORM DISAPPEARANCE IN
RAT AND HUMAN LIVER SLICES[a]

Species	Specimen No.		V_{max} (nmol/min/g)	K_m (nmol/flask)
Rat	RS3		1.90	16.7
	RS2		3.27	13.2
	RS5		3.28	46.6
		$\bar{x} \pm SD$	2.82 ± 0.79	25.5 ± 18.4
Human	H89		2.17	6.3
	H90		2.52	8.7
	H92		4.03	10.0
		$\bar{x} \pm SD$	2.91 ± 0.99	8.3 ± 1.9

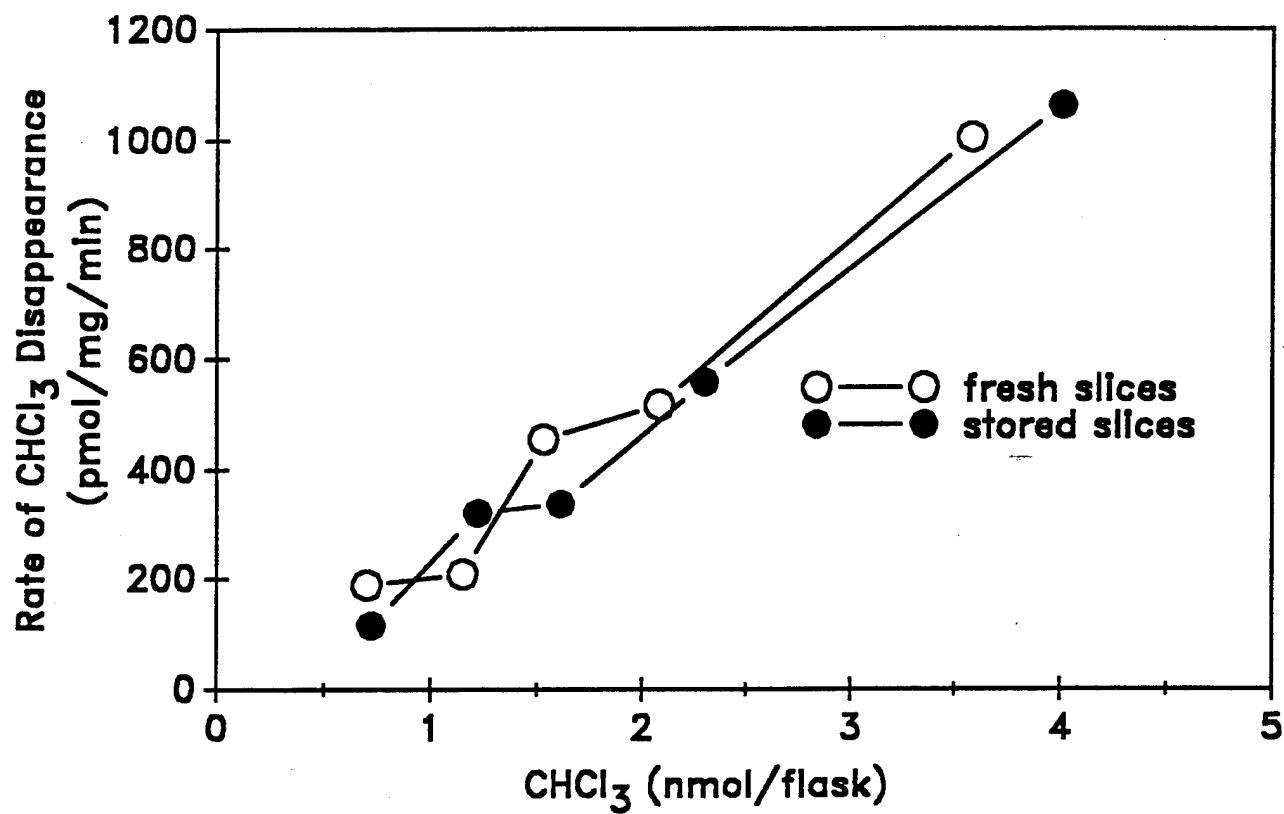[a]The V_{max} and K_m values were calculated from Lineweaver-Burk plots.

Figure 4.   The effect of cold storage on the rate of disappearance of
                 chloroform from the headspace of human liver slice incubations.

CCl$_4$ Metabolism in Rat Liver Slices (5/19/94)
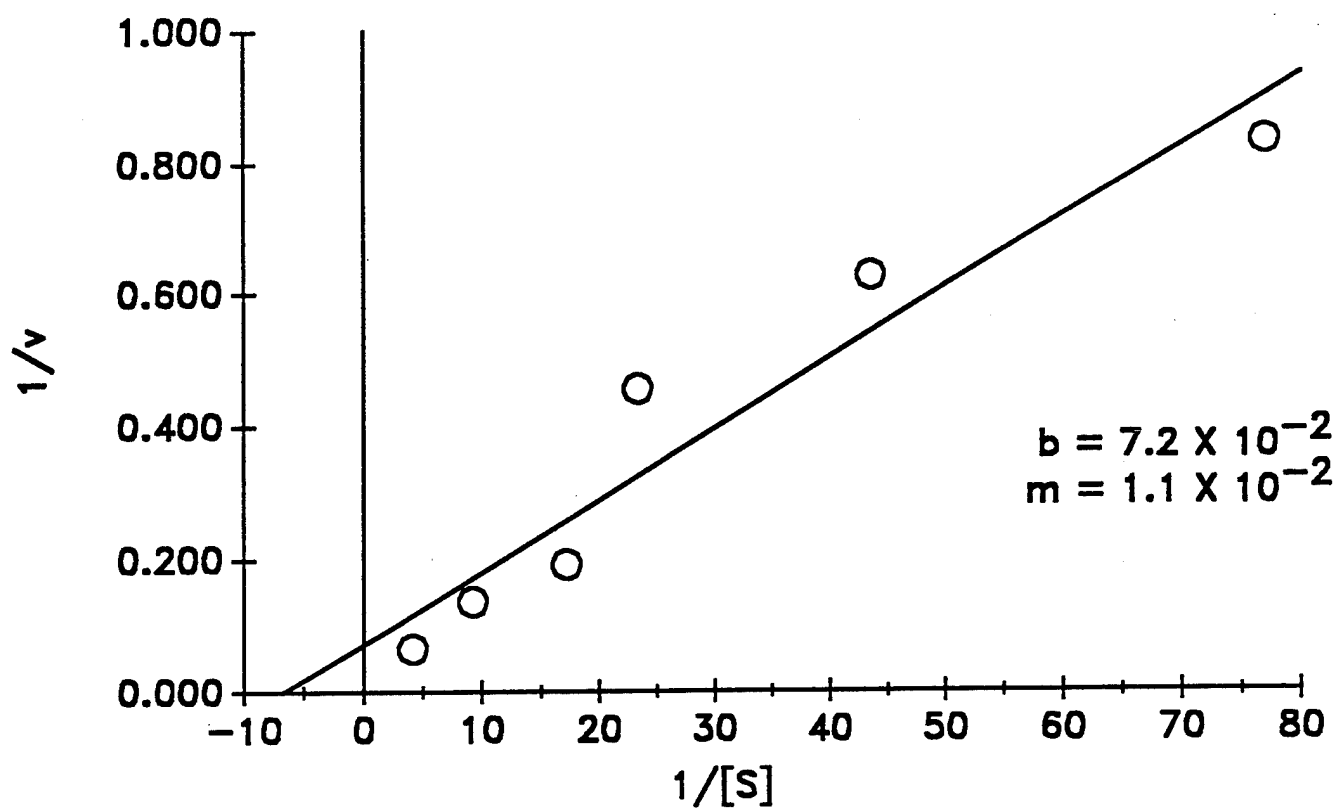
b = 7.2 X 10^{-2}
m = 1.1 X 10^{-2}

Figure 5.   Lineweaver-Burk plots of rat liver slice experiment in which the rate of carbon tetrachloride disappearance from the flask headspace was determined.

CHCl$_3$ Metabolism in Rat Hepatocytes (1/13/94)
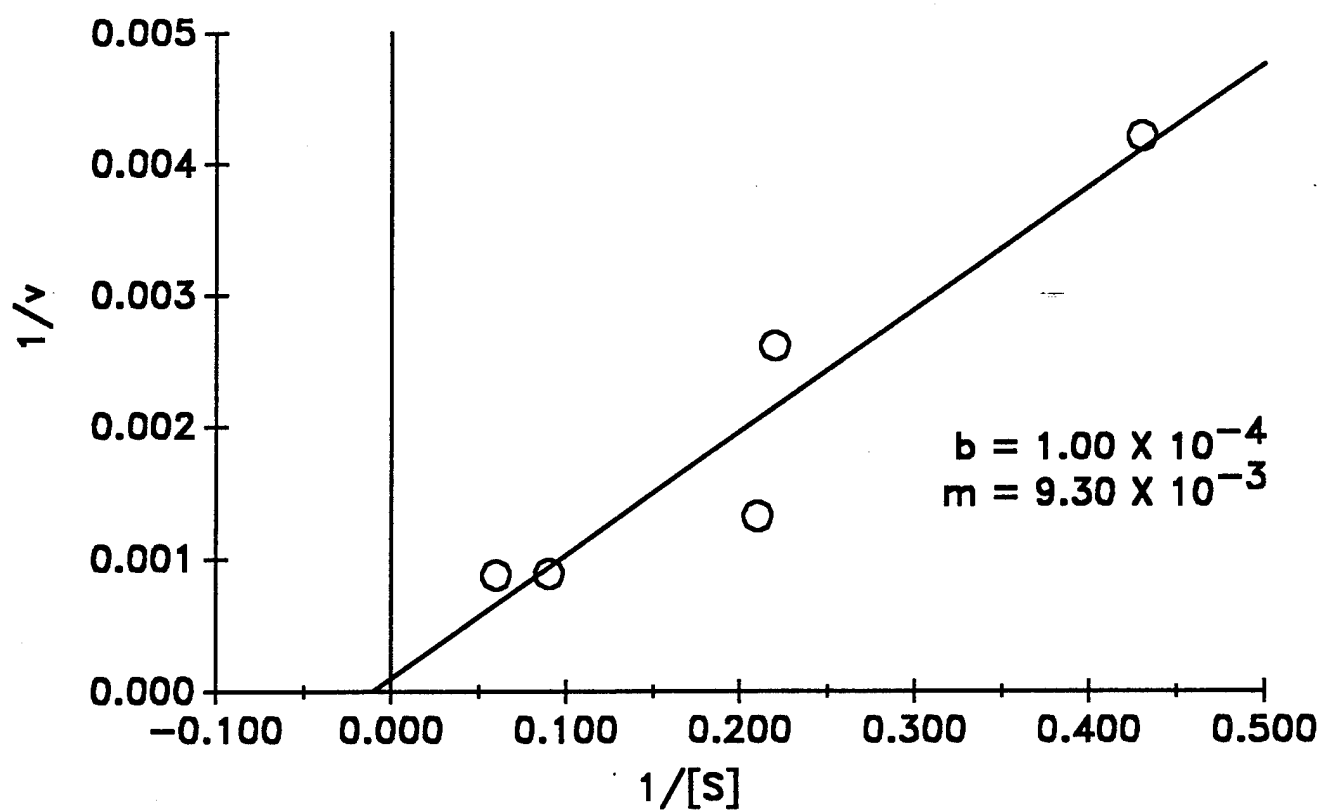
b = 1.00 X 10^{-4}
m = 9.30 X 10^{-3}

Figure 6.   Lineweaver-Burk plot of rat hepatocyte experiment in which the rate of chloroform disappearance from the flask headspace was determined.

FUTURE WORK

The following experiments are planned for the next year of the project:

- Replicate rat hepatocyte experiments with chloroform will be performed to compare with rat liver slice kinetic constants.

- Rat and human liver slice experiments will be continued with carbon tetrachloride to give a total of three replicate experiments with each species.

- Additional chlorinated solvents will be studied in the *in vitro* model as time and the availability of human tissue allow from the list of trichloroethylene, dichloromethane, and bromochloromethane.

We plan to write a complete manuscript describing the experiments with chloroform in hepatocytes and liver slices, comparing the two *in vitro* models, as well as a manuscript in which the metabolism of all the solvents studied in human and rat liver will be compared.

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APPENDIX

Abstract submitted to the 1994 meeting of the International Society for the Study of Xenobiotics

C. E. Green and J. E. Dabbs. Optimization of liver slices for comparing the kinetics of metabolism of chloroform by rat and human liver.

OPTIMIZATION OF LIVER SLICES FOR COMPARING THE KINETICS OF METABOLISM OF CHLOROFORM BY RAT AND HUMAN LIVER.

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The ability to quantitatively compare the metabolism of chemicals using *in vitro* preparations from rodent and human tissues could provide valuable data by improving the risk assessment process for environmental chemicals. In the current study, chloroform was used as a model compound that is metabolized extensively by the liver, to optimize the *in vitro* system. Precision-cut liver slices were prepared using the Krumdieck tissue slicer from adult male Fisher 344 rats and from human organ donors. Slices had a diameter of 9 mm and the thickness was varied such that the weight of the slices ranged from approximately 25 to 150 mg/slice. The liver slices were preincubated, four slices per 2 ml of culture medium, for 60 min in scintillation vials supported on screens in an atmosphere of 95% air:5% CO_2. The slices were then transferred to fresh medium, gassed with 95% air:5% CO_2 and the incubation vials (22 ml total volume) were sealed with a gas-tight valve. Chloroform stock solutions of varying concentrations were added to the vials and the incubations were continued for up to 240 min. Air samples were removed at several timepoints and analyzed by gas chromatography (2 mm x 6 ft glass column packed with 0.1% SP1000 on 80/100 Carbopack C) and electron capture detection. The chloroform concentrations in the headspace remained stable in incubations without slices or with heat-killed slices. Therefore, the loss of chloroform from the headspace was considered to represent metabolism. Initial time course experiments indicated that chloroform was rapidly lost from the vial headspace when incubated with viable rat liver slices; 3.6 nmol/vial completely disappeared by 240 min. The thickness of the liver slices had a dramatic effect on the rate of chloroform metabolism. Rat liver slices weighing 25 mg/slice metabolized chloroform at the rate of 413 pmol/min/g tissue versus slices weighing 150 mg/slice which metabolized chloroform at the rate of 49.5 pmol/min/g tissue, suggesting that metabolism was limited by diffusion into the tissue in the thicker slices. Using the thinnest slices that could be made, the kinetic constants for chloroform metabolism by rat and human liver slices were determined. Three different specimens of each species were studied. With rat liver slices, the Vmax for chloroform metabolism was 2815 ± 794 pmol/min/g tissue and the Km was 25.5 ± 18.4 nmol/vial. With human liver slices, the Vmax of chloroform metabolism was 2907 ± 990 pmol/min/g tissue and the Km was 8.33 ± 1.9 nmol/vial. In summary, the liver slice system was readily adaptable to investigation of the metabolism of a volatile chemical. However, the dramatic effect of tissue slice thickness on chloroform disappearance that was observed suggests that careful optimization of the system is necessary to ensure accurate results. Comparison of the kinetic constants of chloroform metabolism by rat and human liver slices suggested that the rate of biotransformation of this chemical is similar in both species. (Supported by AFOSR)